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An immunological variant of luteinizing hormone (LH) dependent on two point mutations in the gene of the LH β-subunit has increased *in vitro* bioactivity and is detectable in serum with immunofluorometric assays (IFMA). The variant occurs with a frequency of 5-25% in the general population. The altered bioactivity and kinetics of the LH variant are accompanied by changes in LH action and by increased stimulation of the ovarian stromal cells and may be followed by increased production of ovarian testosterone and androstenedione and their estrogen metabolites estradiol and estrone. The study takes advantage of an on-going prospective cohort study of hormones and breast cancer. Serum samples from cohort subjects are being assayed for testosterone, androstenedione, estrone, estrone sulfate, estradiol and SHBG as part of a case-control study nested within the cohort (497 cases and 1,384 controls). In year 1 of the project, we completed a preliminary analysis of the alleged association between breast cancer (cases identified before 1995) and the presence of the variant LH. Preliminary results suggest that a positive association may be present in young, pre-menopausal women. Laboratory analyses of LH variant will be completed in year 2 and the relationship between variant and serum levels of steroid hormones will be examined in the final year.

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Dan Stowe 10/8/1998

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Introduction

It is generally agreed that estrogens and other endogenous hormones are important in the etiology of breast cancer. But no consensus exists as to the precise hormonal environment that characterizes risk or the exogenous factors that influence the hormonal milieu. The vast majority of the epidemiological studies that were conducted in the seventies and eighties were hospital-based case-control studies in which specimen sampling was performed well after the clinical appearance of the disease (1). Evidence from early prospective cohort studies also had limitations in their small sample sizes and/or short follow-up periods. Recent case-control studies within large cohorts (the New York Women's Health Study [2], the ORDET study [3] and the Columbia Breast Cancer Serum Bank Study [4]), suggest that increased estrogens and their androgen precursors are associated with a four- to six-fold increase in the risk of post-menopausal breast cancer. The complex of these data suggests that sex hormones are a key factor in the etiology of breast cancer and that a better understanding of their role is fundamental to developing new opportunities for prevention and control.

The production of ovarian steroid hormones in females is dependent on the secretion of pituitary gonadotropins -- follicle stimulating hormone (FSH) and luteinizing hormone (LH). Recently, an immunologically variant form of the LH β-subunit has been identified and described in the laboratory of one of the co-investigators in the present proposal, Dr. I. Huhtaniemi at the University of Turku, Finland. The variant is detectable with a combination of two ultrasensitive immunoassays using monoclonal antibodies (5). Variations in the detectability of LH with the immunoassay may depend on the structural alteration of the epitopes that are recognized by the monoclonal antibodies and indicate that genetic variants of LH exist in some populations. Two point mutations were described in codons 8 and 15 in the LH β-subunit gene (6,7,8) and pedigree analysis confirmed an autosomal recessive mode of inheritance. In codon 8, a TGG to CGG conversion replaces tryptophan (Trp) with arginine (Arg), and within codon 15 a changing ATC to ACC replaces isoleucine (Ile) with threonine (Thr). The latter substitution is of particular interest because it introduces a potential additional glycosylation site in the LH β-subunit, with the potential for increased bioactivity at the LH receptor site (9). In vitro studies have shown that the variant has increased bioactivity in homozygous subjects as compared to those homozygous for normal LH and the in vivo half-life of the variant LH was shorter than for normal LH (10). These data strongly suggest that some individuals carry a more potent form of LH, though with a shorter life span.

The prevalence of the variant LH β-subunit has been estimated among a broad spectrum of world populations. The carrier frequency of the variant LH beta allele varies from a minimum of 71% in US Hispanics to 41.9% in Lapps of northern Finland (11). The variant appears to increase in frequency in populations of Northern Europe, as compared to those of Asia or from tropical climates. In most European populations outside Scandinavia and in Caucasians in the US the variant frequency is around 15% (12).

The finding of a LH polymorphism with potential increased bioactivity suggests that the variant may correlate with changes in gonadal function. To our knowledge, only one study has addressed the relationship of the presence of the variant LH to clinical and hormonal parameters among women with polycystic ovaries as compared to healthy subjects (10). In this study, the variant was appreciably more frequent in obese women with polycystic ovaries than in normal women. Interestingly, among healthy subjects, women with the LH variant had serum estradiol, testosterone and sex-hormone- binding globulin (SHBG) considerably and significantly more elevated than those

without the variant. These findings provide preliminary evidence strongly suggestive of a different profile of ovarian sex-hormones among subjects with the LH polymorphism.

Methods and Procedures

The New York Women's Health Study

The present study is based on the New York Women's Health Study (NYWHS), an on-going prospective cohort study continuously funded by the National Cancer Institute since 1984 (Principal Investigator, P. Toniolo). The main goal of this project is to assess the role of sex hormones in the etiology of breast cancer, as determined from blood specimens obtained prior to the clinical occurrence of the disease. Between March 1985 and June 1991, the NYWHS recruited in New York City a cohort of 14,275 women, all of whom volunteered to donate for scientific research samples of peripheral venous blood obtained at baseline. Blood specimens from all study subjects (serum, red cells and blood clots), including repeat samples obtained at annual visits, were divided in small aliquots and preserved at low temperature (-80° C) without thawing. At cohort enrollment, 50.3% of subjects were pre-menopausal and 49.7% were post-menopausal. Subjects completed a self-administered questionnaire for demographic, medical, life-style and reproductive information and a semi-quantitative food frequency questionnaire.

The NYUWHS cohort is actively followed-up to identify all incident cases of malignant cancers occurring in the study population. Follow-up is achieved through periodic direct mail contacts with all study subjects and is supplemented by computer linkages with the population tumor registries of the States of New York, New Jersey and Connecticut, and by mortality ascertainment. Most cases are confirmed by review of clinical and pathological documentation. As of June 1997, over 450 cases of breast cancer were identified. A new follow-up (to 2/1998) is under way. With this additional effort, the cohort will have been followed for an average of 12 years. Once the current follow-up is completed, we expect over 1,300 incident cases of malignant tumors, including approximately 620 cases of invasive breast cancer to be identified. Of those, one half will have been diagnosed 5 years or more after baseline blood donation.

Study Subjects

All breast cancer cases and randomly selected non-cancer controls from the same study population are included in a case-control study nested within the cohort. For each case, two controls are selected at random among appropriate risk sets. Risk sets consist of all cohort subjects alive and free of cancer at the time of diagnosis of a given case and matching the case on menopausal status at enrollment, age at entry, date of enrollment, number of blood donations and, if pre-menopausal, day of the cycle at blood donation.

Determination of LH β-subunit variant status

One 1-mL sample of unthawed serum is retrieved from storage for each eligible breast cancer case and their matched controls. Samples are allocated to batches of appropriate size (cases and matched controls are part of the same batch) and are analyzed by the laboratory at the same time. Samples are shipped in dry ice to the laboratory in Finland in the amounts required for immediate analyses and without any information concerning study subject, including matching status.

Laboratory analyses of LH β-subunit variant are performed at the Department of Physiology of the University of Turku, Finland, under the responsibility of Dr. Huhtaniemi. DELFIA LHspec (Wallac OY, Turku, Finland) using two LH β-subunit specific monoclonal antibodies (Mab) serve as reference method (assay 2). This assay detects with equal stochiometry the wild-type and variant form of LH. In the other assay (assay 1), the capture Mab recognizes a conformational epitope present in the intact α/β LH dimer but not in the subunits, and the detection Mab recognizes an epitope in the α-subunit. The capture Mab of this assay only recognizes the wild-type hormone.

The ratio of LH values measured by the two assays (assay1/assay2) determines the variant/wild-type LH status. Three separate categories of this ratio are obtained: 1) normal ratio (>0.9; two wild-type LH alleles); 2) low ratio (0.2-0.9; heterozygous for variant LHβ gene); 3) zero ratio (below 0.15; homozygous for the variant LHβ gene). The sensitivity of the two IFMA is 0.05 IU/L, and the intra- and inter-assay coefficients of variations are less than 4% and 5%, respectively, at LH concentrations at and above the lowest standard concentration (0.6 IU/L of WHO 80/552). These analyses were validated against those obtained by sequencing the LH β gene: there is full agreement between the two methods, and both point mutations are found in each individual sample studied.

Interim Results and Discussion

In year 1, we conducted two small preliminary studies to clarify specific issues to fine tune study design and management. Later in the year, we completed the first round of laboratory measurements as planned.

First preliminary study. The goal was to determine how well laboratory measurements would predict LH variant status in single samples. The reliability of measurements of LH variants was assessed in the same individual at different sampling times. From the NYWHS database, 20 subjects were identified who had donated samples of blood on two separate occasions. We confirmed that the classification of individuals into categories of LH variants (wild type, heterozygous, homozygous) corresponded exactly in each determination.

Second preliminary study. LH variant measurements were compared in samples that have been stored without ever being thawed and in those that had undergone repeated, complete defrosting. This was useful, as the NYWHS maintains many specimens that are returned from laboratories after the completion of analyses. We compared thawed vs. unthawed samples from 10 subjects and confirmed that the measured values remained unchanged. We concluded that previously thawed samples could be used in place of never thawed ones in subsequent analyses.

First phase of the nested case-control study. The first batch of LH variant analyses were completed at the laboratory of Dr. Huhtaniemi in Finland in September 1998. The results presented here should be considered preliminary, as we intend to avoid conducting interim analyses before the completion of the study as planned.

After excluding cases of breast cancer diagnosed within 6 months of diagnosis, 388 cases confirmed by review of clinical and pathological records were included in the first batches of laboratory analyses. Of the 776 control subjects initially selected, one was excluded because her hormonal assays were done in a different batch than those for the matching case subject. As a result, 388 breast cancer cases (322 invasive and 66 non-invasive) and 775 controls were considered. Selected characteristics of the study group are given in Table 1. The majority of study subjects (69%) were Caucasian, 8% were African-American, and 3% were Hispanic. Approximately one third of the subjects were Jewish (37% of case subjects and 35% of controls), 21% were Catholic and 15% were Protestant. This ethnic

composition reflects the characteristics of the patient population at the screening clinic at the time of recruitment. The median age at diagnosis of breast cancer was 57.2 years and the median duration between initial blood donation and diagnosis was 2.2 years. There were no significant differences between breast cancer cases and controls in age at menarche, parity, and age at first full-term pregnancy. Compared to controls, case subjects were more likely to report a prior benign breast condition and to have a history of breast cancer in first degree relative.

Table 1. Characteristics of study subjects

Characteristic	Breast Cancer Cases (n = 388)	Controls (n = 775)
Age (years) at blood donation, median (range)	54.5 (34.0-68.0)	55.0 (34.0-68.0)
Age (years) at diagnosis, median (range)	57.2 (35.4-71.4)	
Age (years) at menarche, median (range)*	12 (9-17)	13 (8-18)
Ever pregnant (%)*	78.4	80.4
Age (years) at first full-term pregnancy, median (range)	25 (16-41)	24 (15-43)
Breast cancer in first degree relative (%)	9.8	6.8
Prior benign breast condition (%)*	61.1	52.1
Height (cm), median (range)*	163 (147-183)	163 (145-183)
Weight (kg), median (range)*	66 (41-123)	64 (40-141)
Quetelet's index (kg/m^2), median (range)*	24.98 (17.01-43.58)	24.22 (16.94-55.08)

* A few missing values

The average normal ratio of LH measured by IFMA assay 1 to that measured by IFMA assay 2 was 1.3 (range 1.0-2.63) if both alleles of the LHβ gene are normal. Low assay 1/assay 2 ratio of 0.6 (0.33-0.98) indicated a heterozygous individual with one normal and one variant allele. Assay 1/assay 2 ratio close to zero (< 0.1) was characteristic for subjects with two mutated LHβ alleles (variant LH homozygotes) (8). Out of 1163 NYUWHS subjects included in the analysis, 153 had low assay 1/assay 2 LH ratio (144 heterozygous and 9 homozygous subjects) which resulted in the variant LH prevalence rate of 13.2%. Table 2 shows characteristics of normal LH and variant LH subjects from NYUWHS cohort. Among 388 breast cancer cases included in the analysis, 333 had normal and 55 had variant LH.

Table 2. Characteristics of normal and variant LH subjects, New York University Women's Health Study, 1985-1990

Characteristic	Normal LH Subjects (n = 1010)	Variant LH Subjects (n = 153)
Age (years) at blood donation, median (range)	55 (34.0-68.0)	50.0 (34.0-65.0)
Age (years) at diagnosis, median (range)	57.4 (35.4-71.4)	54.0 (38.6-68.8)
Age (years) at menarche, median (range)*	13 (8-18)	12.5 (10-16)
Ever pregnant (%)*	79.7	80.0
Age (years) at first full-term pregnancy, median (range)	24 (15-43)	24 (16-41)
Breast cancer in first degree relative (%)	8.0	6.5
Prior benign breast condition (%)*	55.3	53.9
Height (cm), median (range)*	163 (145-183)	163 (147-183)
Weight (kg), median (range)*	64 (41-141)	64 (40-109)
Quetelet's index (kg/m^2), median (range)*	24.2 (17.0-54.8)	24.1 (17.5-39.9)

* There were a few missing values

Breast cancer cases with variant LH tended to be younger at diagnosis than cases with normal LH (57.4 vs. 54.0) ($p = 0.08$). As compared to subjects with normal LH, variant LH carriers reported less frequent breast cancer in first degree relatives (6.5% vs. 8.0%) and similar age at menarche, parity, age at first full-term pregnancy, height, weight, and Quetelet's index.

The distribution of normal and variant LH in breast cancer cases and controls is shown in Table 3. The frequency of variant LH among breast cancer cases was only marginally higher than among controls (14.1% vs. 12.6%) ($p = 0.47$).

Table 3. Luteinizing hormone status of breast cancer cases and controls, New York University Women's Health Study

LH Status	Breast Cancer Cases (n = 388)	Controls (n = 775)
Normal LH (wild-type)	333 (85.8%)	677 (87.4%)
Variant LH (heterozygous)	51 (13.1%)	93 (12.0%)
Variant LH (homozygous)	4 (1.0%)	5 (0.6%)

Overall, there was an initial suggestion of a positive association between LH variant carriers and increased risk of breast cancer, but the association appeared to be more evident among women with cancer diagnosed at young age (<50).

Recommendations in Relation to the Statement of Work

Technical Objective 1: Association between LH variant status and breast cancer

The technical objective was reached only in part, owing to the delayed completion of the follow-up in the parent project and, thus, the incomplete identification of the most recently diagnosed breast cancer cases in the cohort. The follow-up is now reaching completion and we expect that the objective will be ultimately met without delays before the end of year 2. About one third of all laboratory analyses remain to be completed before conducting a full statistical analysis.

All preparatory work described in the original Statement of Work has been completed as planned. The only relevant difference was a slightly smaller sample size for the preliminary assessment of LH variant status in the cohort (20 subjects, two samples each, instead of 40 subjects, two samples each), dictated by the need to avoid the unnecessary use of irreplaceable specimens. A further change was represented by the addition of a preliminary study on variant status assessment after defrosting, which demonstrated that defrosting had no effect on a large protein such as LH.

Technical Objective 2: relationship between LH variant status and serum levels of ovarian steroid hormones

This objective will be addressed in year 2-3, as planned.

Conclusions

The project has already completed a large portion of its stated objectives. It will continue in year 2 with the completion of all laboratory analyses of LH variant status and the creation of a larger database containing the result of laboratory analyses of endogenous hormones performed in the parent project.

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